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Expression of Three Alternative Acetylcholinesterase Messenger RNAs in Human Tumor Cell Lines of Different Tissue Origins

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To study the molecular mechanisms underlying the intensive expression of acetylcholinesterase (AChE) in different tumor types, we characterized levels and composition of its messenger RNA (mRNA) sequences in heterologous tumor cell lines, primary tumor biopsies, and normal fetal and adult tissues and determined their exon–intron origin within the corresponding ACHE gene. Reverse transcription followed by polymerase chain reaction (RT–PCR) revealed three alternatively spliced ACHE mRNAs in NT2/D1 teratocarcinoma, NCI-N-592 small cell lung carcinoma, TE671 medulloblastoma, K-562 erythroleukemia, and 293 transformed embryonal kidney cells. The three ACHE mRNAs include the principal species expressed in brain and muscle and two additional transcripts containing insertions of 751 or 829 residues downstream from the exon 4 domain. The inserted region, which represents an intron in brain and muscle, is expressed in the tumor cell lines either as a "readthrough" form or with 78 residues deleted from its 5′ end. A major band of 2.5 kb was labeled with ACHE cDNA in poly(A)⁺ RNA blots from medulloblastoma cells or brain tissue, whereas a PCR-amplified probe from the inserted domain labeled a 3.4-kb band but not the 2.5-kb band in poly(A)⁺ RNA from small cell lung carcinoma. The ACHE mRNAs including the alternative insertions were found only in cell lines with levels of the principal ACHE mRNA species equal to or higher than those in brain (1–10 molecules/cell), determined by following the kinetics of mRNA PCR amplification. Genomic DNA sequencing revealed that the inserted domains in the ACHE mRNAs expressed in the tumor cell lines encode C-terminal peptides of 40 and 14 residues. These include a free cysteine, terminate with the consensus HG element, and continue by a 29-residue-long C-terminal hydrophobic cleavable peptide, properties characteristic of precursors to phosphoinositide (PI)-linked proteins. In extention of the reported expression of PI-linked AChE in hemopoietic cells including K-562, our findings demonstrate the existence of ACHE mRNAs with the potential to encode one hydrophilic and two PI-linked forms of AChE in tumor cells from both hemopoietic and nonhemopoietic origins. © 1994 Academic Press, Inc.

INTRODUCTION

The ubiquitous acetylcholine hydrolyzing enzyme acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7) acquires heterogeneous properties in different tumors [reviewed in Refs. 1, 2], distinct from those it displays in muscle and nerve [for reviews see Refs. 3, 4], hemopoietic cells [5, 6], embryonic tissues [7], and germ cells [8]. Monomers of the catalytic AChE subunit were observed in meningiomas and tetramers in glioblastomas [9], and inhibition properties different from those of normal AChE were determined for serum AChE in various carcinomas [10]. Moreover, tumorigenic expression of the corresponding ACHE gene was found to be subject to variable control mechanisms. In differentiating neuroblastoma cells, inhibition of mevalonate synthesis, which decreases proliferation rates, increases AChE levels [11]. In PC12 cells, in contrast, nerve growth factor induces the production of hydrophilic AChE [12], while embryonal carcinoma cells and thyroid tumor cells produce this enzyme under all conditions examined [13, 14].

A major hydrophilic form of AChE with the potential to be "tailed" by noncatalytic subunits is expressed in brain and muscle [15, 16], whereas a hydrophobic, phosphoinositide (PI)-linked form of the enzyme is found in erythrocytes [17]. Two sublines of the human erythroleukemic K-562 cells were shown to express the PI-linked form of AChE, however, with different structural properties of the PI moiety [18]. To reveal the molecular mechanisms underlying the heterogeneous tumorigenic expression of AChE, we initiated the investigation of

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alternative splicing in ACHE messenger RNAs (mRNAs) from different tumor cells.

Alternative splicing controls the generation of proteins with diverse properties from single genes [19], through the alternate excision of intronic sequences from the nuclear precursors of the relevant mRNAs (pre-mRNA). It is known to be cell type-, tissue-, and/or developmental stage-specific [20] and is considered the principal mechanism controlling the site(s) and timing of expression and the properties of the resultant protein products from various genes [21]. The specificity of alternative splicing depends on the availability of splicing factors and/or repressor proteins that bind to pre-mRNA recognition sequences and control the selection of particular splice sites [19]. In view of the polymorphism of AChE forms in different tumor tissues and cell types, the alternative splicing pattern of the ACHE gene in these cells is therefore of special interest. This, in turn, depends on the exon–intron organization of this human gene.

Alternative exons encoding the C-terminal peptide in AChE were shown to provide the molecular origins for the amphiphilic (PI)-linked and the hydrophilic "tailed" form of AChE in *Torpedo* electric organ [22, 23]. The existence of parallel alternative exons [16] and homologous enzyme forms in mammals [24, 25] suggested that a similar mechanism may provide for the molecular polymorphism of human AChE. However, the only cDNAs reported to date from mammalian brain and muscle encode the hydrophilic AChE form [15, 26]. Nonetheless, RNA protection and polymerase chain reaction (PCR) analyses have demonstrated the existence of two rare alternative ACHE mRNAs in mouse hemopoietic cells [16], raising the question whether a similar situation prevails in human cell lineages. To this end, we performed RT-PCR analyses and RNA blot hybridizations in normal and tumor tissues and determined the nucleotide sequence in the corresponding domain within the ACHE gene. Our findings reveal in different tumor cell lines the expression of two alternative ACHE mRNA species, in addition to the major species expressed in brain and muscle, and suggest the presence of three distinct forms of AChE, two of which may be PI-bound to the cell surface, in several types of malignant cells.

MATERIALS AND METHODS

Cell lines, tumor biopsies, and tissue sources. The NT2/D1 teratocarcinoma cells were grown as in [27]. The H9 T cell lymphoma, IMR32 neuroblastoma, 293 embryonal kidney cells, and TE671 medulloblastoma cells were received from the American Type Culture Collection and grown according to the instructions provided. NCI-N-592 small cell lung carcinoma cells were grown as detailed elsewhere [28]. The hemopoietic cell lines K-562, HL60, and DAMI [29] were gratefully received from E. Kedar (Ein Kerem), Y. Yardan (Rehovot), and A. Eldor (Ein Kerem), respectively. Tumor biopsies from serous ovarian adenocarcinoma, mucinous cyst adenoma, papillary adenocarcinoma, and benign myoma were removed at surgery in the Department of Obstetrics and Gynecology at the Edith Wolfson Medical Center and were pathologically characterized according to established procedures. Control tissues from fetal and adult individuals were obtained as previously detailed [30].

cDNA and genomic clones and DNA sequencing. The genomic GNACHE clone and the HACHE recombinant transcription construct were as described [15]. Deletion of 94 bp from HACHE at position 1616–1710 by *Bam*HI excision and religation created the control plasmid HdACHE for the quantitative RT-PCR experiments.

Double- and single-stranded DNA were sequenced as detailed previously [15], except that annealing of ACHE-specific sequencing primers was initiated at 72°C, to circumvent the high GC-rich content of the analyzed sequences. Also, sequencing primers were closely spaced, and reading included comparison of sequence data from both directions and with variable distances from the primers employed.

PCR primers and the RT-PCR procedure. The following PCR primers were employed:

(1) 1522(+): 5'-CGGGTCTACGCCTACGTCTTTGAACACCGTGCTTC-3'
(2) E6/2003(−): 5'-CACAGGTCTGAGCAGCGATCCTGCTTGCTG-3'
(3) I4/1939(−): 5'-GGTTACACTGGCGGGCTCC-3'
(4) E5/1917(−): 5'-ATGGGTGAAGCCTGGGCAGGTG-3'
(5) E5/1900(+): 5'-GCCCAGGCTTCACCCAT-3'
(6) 1281(+): 5'-AGACTGGGTAGATGATCAGACACCTGAAAACTACCG-3'
(7) 1635(−): 5'-GACAGGCCAGCTTGTGCTATTGTTCTGACTCTCAT-3'
(8) 1565(+): 5'-ACCGTCCACCTGAACTGCTACTGGGAGAAG-3'
(9) 1887(−): 5'-CGCTTACTAGGATCCAAGGCAAGCATGTAA-3'
(10) E5/2519(−): 5'-AGAAATGCAGGCGACCACGTG-3'.

For the positions of primers 1–5, 10 along the ACHE gene, see Fig. 3. Butyrylcholinesterase (BCHE, primers 6 and 7) and CHED primers (8 and 9) were numbered according to previously published sequence data [31, 32, respectively].

For RT-PCR analyses, total RNA was extracted by the guanidinium thiocyanate method as described [15]. Random hexamer primers (Boehringer, Mannheim, Germany) were employed for cDNA preparation from 0.1 μg RNA of each sample using the MMLV reverse transcriptase (rt) (Gibco, BRL, Bethesda, MD), essentially as described elsewhere [32]. PCR amplification in the 9600 thermal controller (Perkin–Elmer/Cetus, Norwalk, CT) (39 cycles) was performed using the noted primer pairs as follows: denaturation 94°C, 1 min (first step 2 min); annealing 65°C, 1 min; and synthesis 72°C, 1 min (last cycle 6 min). Amplification products (20%) were electrophoresed (7 V/cm, 60 min) on 1.6% agarose (IBI, CT) gel containing ethidium bromide (0.5 μg/ml, Sigma) with TAE (40 m*M* Tris–acetate, 2 m*M* EDTA) as electrophoresis buffer and were photographed under 320-nm illumination. Control reactions, without rt, remained negative, proving the absence of contaminating DNA sequences corresponding to the relevant mRNA sequences.

Quantification of ACHE mRNA levels. RT-PCR amplification was performed in 100-μl reactions as detailed above, except that 10-μl aliquots were sampled every third cycle from cycle 21 on. Following agarose gel electrophoresis and photography, the intensity of fluorescence in the DNA bands at each time point was densitometrically determined [33] and the percentage of maximum intensity was calculated at each kinetic follow-up. HdACHE mRNA (10^5–10^7 copies), *in vitro* transcribed as described [15] and purified by double DNaseI digestion, was subjected to similar procedures and served for calibration.

RNA blot hybridizations. Poly(A)$^+$ mRNA from the noted tissue and cell sources was prepared using poly(dT) Dynabeads (BRL, Gaithersburg, MD) according to the producer's instructions. Electrophoresis (10 μg/lane), blotting, and hybridization with ^{32}P-labeled ACHE cDNA or PCR-amplified probes were as described [34]. Exposure was for 5–10 days with CAWO intensifying screens.

RESULTS

Alternative Intron Excisions

Sequencing of the cloned human ACHE [15, 35] gene revealed a 829-bp-long domain which operates as an intron between exons 4 and 6 [for nomenclature see Ref. 36] and is spliced out in the ACHE mRNA form expressed in brain and muscle [15]. Sequence analysis demonstrated the presence of the consensus splicing motifs GT (at position 11) and AG (at position 87) with a preceding pyrimidine stretch. This implied that nucleotides 11–87 in this region constitute an intron, designated I4, whereas the remaining sequence (nucleotides 88–839) represents an additional exon, designated E5. The human I4 intron constitutes an open reading frame (ORF) continuous with that of both E4 and E5 (Fig. 1A). The ORF in E5 was found to encode a polypeptide with a potential for cleavage and subsequent linkage of a phosphoinositide moiety [37], yet shares no homology with the *Torpedo* 3H alternative exon located at a similar position [23]. The nucleotide sequence in the short ORF region from E5 was identical to that in a previous report [16] except for a single nucleotide difference at position 159 (from G to C), implying a single amino acid substitution (Pro instead of Arg) in the 18th amino acid residue of the E5 peptide. This difference reflects natural polymorphism [38]. The remaining 530 bp of E5 were fully sequenced and found to be nontranslatable (Fig. 1B). When the I4 + E5 domain was introduced into the HACHE plasmid [35] instead of the E6 region, catalytically active enzyme was produced in microinjected *Xenopus* oocytes (data not shown), demonstrating that the product of these alternative transcripts is fully functional.

Tumorigenic expression and 3′ splice options in the coding domain were first examined by RNA blot hybridization, in the search for full-length ACHE mRNA transcripts. Hybridization with the brain ACHE cDNA probe [15] revealed a single 2.5-kb band for poly(A)$^+$ ACHE mRNA from TE671 medulloblastoma (Fig. 2A) and fetal and adult brain (not shown). A faint 28S band, apparently nonspecific labeling of ribosomal RNA, was found in both TE671 and NCI-N-592 cells. There was no labeling in IMR32 RNA, indicating low levels of intact ACHE mRNA transcripts (Fig. 2A). Signals obtained with 10 μg poly(A)$^+$ RNA from all of these sources were lower than those observed for 1 ng of *in vitro* transcribed ACHE mRNA (not shown), demonstrating that ACHE mRNA constitutes less than 1:10^6 of total RNA and in agreement with library screening studies [15]. RNA blot hybridization was further performed with a selective probe from the E5 genomic domain. This probe did not label brain ACHE mRNA, demonstrating specificity (not shown). In the NCI-N-592 carcinoma cells, it labeled a band of 3.4–3.5 kb as well as the 5.1-kb 28S ribosomal RNA (Fig. 2B). A nonspecific band of ca. 1.5 kb, far shorter than the coding sequence of ACHE mRNA, was also labeled in both NCI-N-592 and IMR32 cells. No labeling in the TE671 lane suggested a low level of the intact E5-containing transcript or its total absence (Fig. 2B). Thus, the RNA blot hybridization data indicated variably efficient tumorigenic expression of at least two alternative ACHE mRNA species, the previously characterized brain and muscle transcript and a larger one, which includes the E5 domain.

To further analyze expression levels and exon–intron boundaries in the alternative tumor ACHE mRNA transcripts, the highly sensitive method of PCR amplification was employed. To this end, PCR primers from the E3, I4, E5, and E6 domains were used with reverse-transcribed cDNA preparations from various cells and tissues (Figs. 3A and 3B). These experiments reconfirmed the presence of a single ACHE mRNA species from which the I4 + E5 domain was spliced in brain and muscle and revealed two additional splicing patterns in ACHE mRNA from tumor cells (Figs. 3C and 3D and Table 1). PCR primers designed to detect E5 demonstrated the presence of an ACHE mRNA species including this exon in NT2/D1 teratocarcinoma, 293 transformed embryonal kidney cells, NCI-N-592 small cell lung carcinoma, TE671 medulloblastoma, DAMI promegakaryoblastic cells, and K-562 erythroleukemic cells (Fig. 3 and Table 1). In all cell types except small cell lung and teratocarcinoma, the PCR band reflecting the alternatively spliced ACHE mRNA was considerably less intense than that representing the brain species, which may reflect low abundance of this mRNA in the tumor cells. Interestingly, we further observed the unspliced I4–E5 "readthrough" transcript reported in murine bone marrow cells [16] in all cell lines expressing the E5 alternative exon. PCR reactions detecting the E5–E6 connection were positive in all cell lines expressing E5 and were relatively intense in teratocarcinoma (Fig. 3C and Table 1). Thus, three ACHE mRNA species were predicted (Fig. 3E).

Since splicing requires precise matching of the terminal nucleotides within each domain, folding energy values were examined (The FOLD Program, University of Wisconsin). These should display considerable differences if they determine specific splicing events. However, the observed values of Gibbs free energy for the E3 and E5 exons were indistinguishable (185 and 180 kcal/

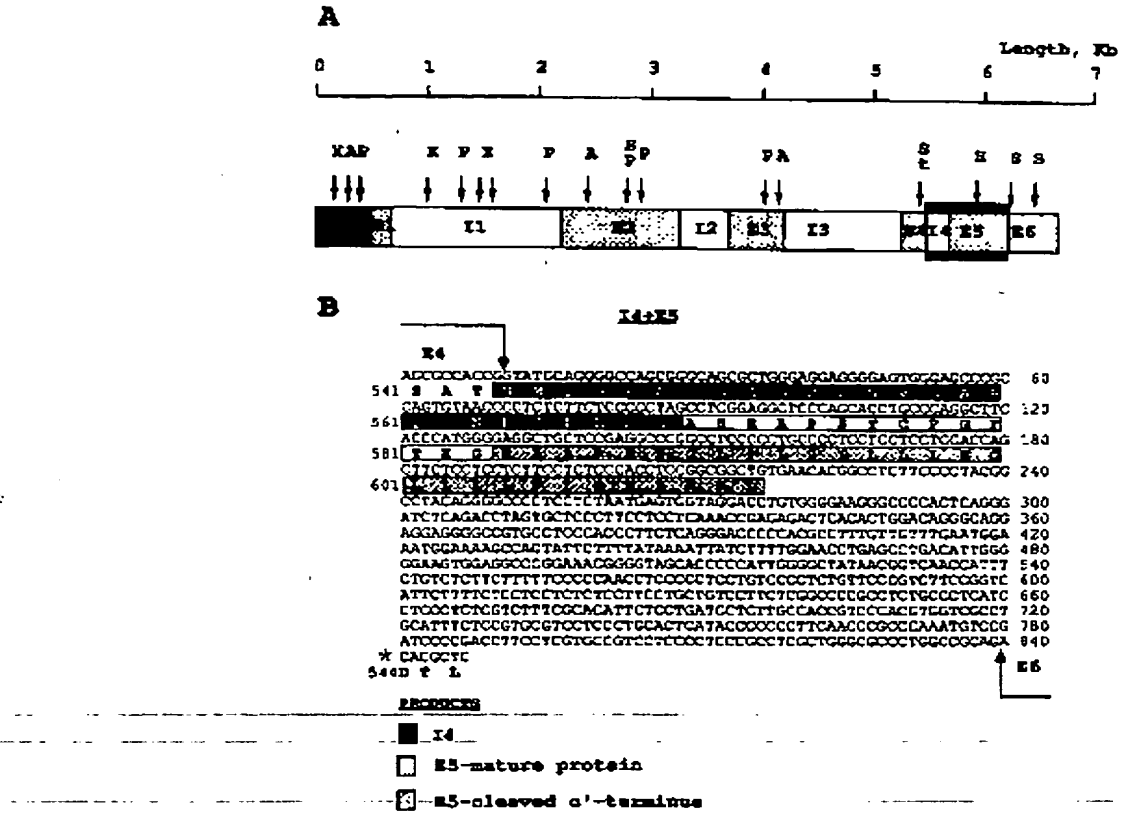

FIG. 1. The human ACHE gene structure and sequence of the I4–E5 domain. (A) Gene structure and restriction sites. The human ACHE gene includes a promoter region (black), six exons, designated in this scheme E1–E6 (shaded boxes), and four introns, I1–I4 (empty boxes), the sizes of which are noted on the length scale in kilobases (kb) where the first sequenced nucleotide equals 0. Lengths of introns and characteristic restriction sites (S, *Sac*I; X, *Xho*I; A, *Acc*I; K, *Kpn*I; P, *Pvu*II; SP, *Sph*I; St, *Stu*I; H, *Hin*cII) within clone GNACHE [15] were determined as detailed elsewhere [35]. The I4–E5 domain is framed in black. (B) Nucleotide sequence of the I4–E5 domain and open reading frame. DNA sequence is presented for the I4–E5 domain. Nucleotides are numbered at the right-hand side. Analysis of this region using the MAP program (University of Wisconsin) revealed a continuous open reading frame connecting the E4, I4, and E5 regions. The predicted polypeptide diverts from the common AChE sequence at amino acid residue 544 and is numbered accordingly on the left-hand side. The I4-translated peptide (white letters on black background), E5 contribution to the mature protein (black letters on white background), and the cleavable C-terminal peptide (shaded) are presented. E4 and E6 splice sites are marked by arrows. Starred amino acid residues are translated from the E6 domain.

mol, respectively), predicting no preference for E5 splicing over other patterns. Thus, selective protein factors should either favor or prevent the inclusion of E5 in ACHE mRNA transcripts. Further experiments would be required to reveal if the various ACHE mRNA and/or AChE forms differ in their stability.

Quantitative ACHE mRNA Evaluation

To evaluate the level of ACHE mRNA within the analyzed cells, RNA extracted from these cell types was subjected to reverse transcription coupled with kinetic follow-up of PCR amplification. For this purpose, we used PCR primers 1 and 2, which detected only the ACHE mRNA species containing E3, E4, and E6 and gave rise to a 481-bp PCR fragment. For calibration, we used measured amounts of HdACHERNA, *in vitro* transcribed from the HdACHE deletion construct of human ACHE cDNA. These deleted ACHERNA molecules, when subjected to RT–PCR amplification using the same set of ACHE primers, produced a 387-bp PCR fragment, easily distinguishable from the natural fragment (Fig. 4A). Densitometric analysis of the electrophoretically separated fragments, stained with ethidium bromide, revealed that the timing of the appearance of fluorescent PCR products depended on the number of ACHE mRNA copies employed. This was also the case when similar amounts of the native and the deleted fragments were amplified together (not shown). ACHE mRNA products from 100-ng samples of teratocarcinoma RNA were reproducibly detected earlier than those from similar amounts of adult brain RNA (Figs.

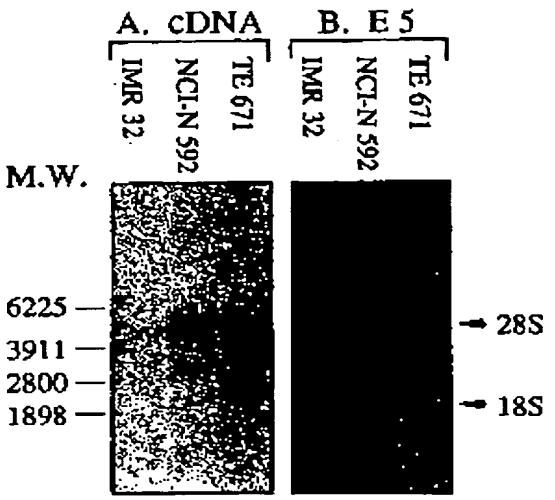

FIG. 2. RNA blot hybridization. (A) Tumor ACHE mRNA detected with the ACHE cDNA probe. Poly(A)$^+$ mRNA samples (5–10 μg/lane) were prepared from cultured IMR32, NCI-N-592, or TE671 cells. Hybridization was with a ^{32}P-labeled brain ACHE cDNA probe [15], produced by enzymatic digestion from plasmid DNA, electroeluted from the gel, and column purified. Washing was performed for 1 h in 0.30 *M* NaCl, 0.022 *M* sodium citrate, 0.5% sodium dodecyl sulfate at 65°C. Electrophoretic migration of 28S and 18S ribosomal RNA and known size markers (Boehringer, Mannheim) is marked. (B) Selective detection of tumor ACHE mRNA transcripts including E5. RNA blot hybridization was as in A, using a PCR-amplified E5-specific probe prepared with primers 5 and 10 (see Materials and Methods) using clone GNACHE as a template. The probe was gel-eluted and random-prime labeled as detailed elsewhere [16].

4A and 4B), demonstrating concentrations 10-fold higher than those in brain (10^7 compared with 10^6 molecules/μg RNA, respectively). Based on our average yields of 1 μg RNA/mg wet wt tissue, and assuming ca. 1 × 10^6 cells/mg tissue, this implies 10 and 1 ACHE mRNA molecules/cell, on the average, or 20 and 2 p*M* concentrations of this mRNA in teratocarcinoma cells and adult brain, respectively.

ACHE Gene Expression in Various Tumor Cell Lines

RT–PCR was employed to quantify ACHE mRNAs in tumor cell lines of different tissue origins, primary tumor biopsies, and normal tissues (Table 1). The major brain species of ACHE mRNA was detected in all of the cell lines examined, with the exception of the H9 T-cell lymphoma and the lymphocytic HL60 cells (not shown) and in line with a previous report of BCHE but not ACHE expression in a lymphocyte cell line [39]. Six of the nine tumor cell lines examined further expressed the alternative E5 exon with different efficiencies. The product representing the readthrough species was particularly bright in at least one of these cell types and was clearly observed in four more lines. All of the ACHE mRNA preparations containing E5 also exhibited a direct connection to E6 (Table 1 and unshown data). However, we cannot exclude the possibility that part of the ACHE mRNA transcripts in these preparations lacked the E6 domain.

In contrast with the expression of E5-containing transcripts in tumor cell lines, we could not detect any ACHE mRNA other than the major brain transcript in primary tumor biopsies, including three types of malignant ovarian adenocarcinomas and benign myoma (Table 1), nor did we find it in five different samples from malignant brain tumors (not shown). In RNA preparations from fetal and adult brain and from adipose tissue, we could detect only the major ACHE mRNA species. RT–PCR amplification of mRNAs encoding the AChE-homologous enzyme BChE [31] and the cell division controller CHED [32] served to verify the integrity of the examined RNA preparations.

Variable Translation Products

The open reading frames in the I4 and E5 domains imply potential changes in the AChE protein products of the alternative transcripts. Because of the nontranslated part of E5, the open reading frame in E6 will not be translated in E5-containing transcripts. Therefore, our data predict that in several tumor cells three different C-terminal peptides may stem from the E6, E5, or I4/E5 domains in the ACHE gene. The inferred AChE forms divert from each other at the amino acid (aa) position 544 [15, 35], and the peptide translated from the I4/E5 region and presented in Fig. 1B is absent in the 583-aa-long hydrophilic "tailed" brain AChE form, encoded by exons 2, 3, 4, and 6. The predicted phosphoinositide-linked AChEs produced from the E5-containing transcripts should be 583 and 557 aa long, with their 40 and 14 C-terminal amino acids translated from the open reading frames in the alternative I4 + E5 or E5 domains, respectively. Yet 29 more residues, also translated from the E5 exon, constitute a hydrophobic cleavable peptide common to both AChE forms produced from E5-containing transcripts. When they appear after the HG dipeptide, such hydrophobic domains are characteristic of precursors to PI-linked proteins [36, 40]. Our *Xenopus* microinjection experiments revealed that at least two of these three AChE protein forms should be produced in various tumor cells. Figure 5 presents the C-terminal peptides characteristic of the alternative human AChE forms compared with their rat counterparts [41, 42]. Interestingly, the C-terminal peptides of the hydrophilic AChE form are virtually identical in rat and human, with the exception of a minor alteration of one amino acid residue (replacement of aspartate 578 in the human enzyme by glutamate in rat). The human E5 inferred translation product presents a more limited 53% identity with the rat product, whereas the homology within the I4 inferred products was found to be negligible (8%, Fig. 5).

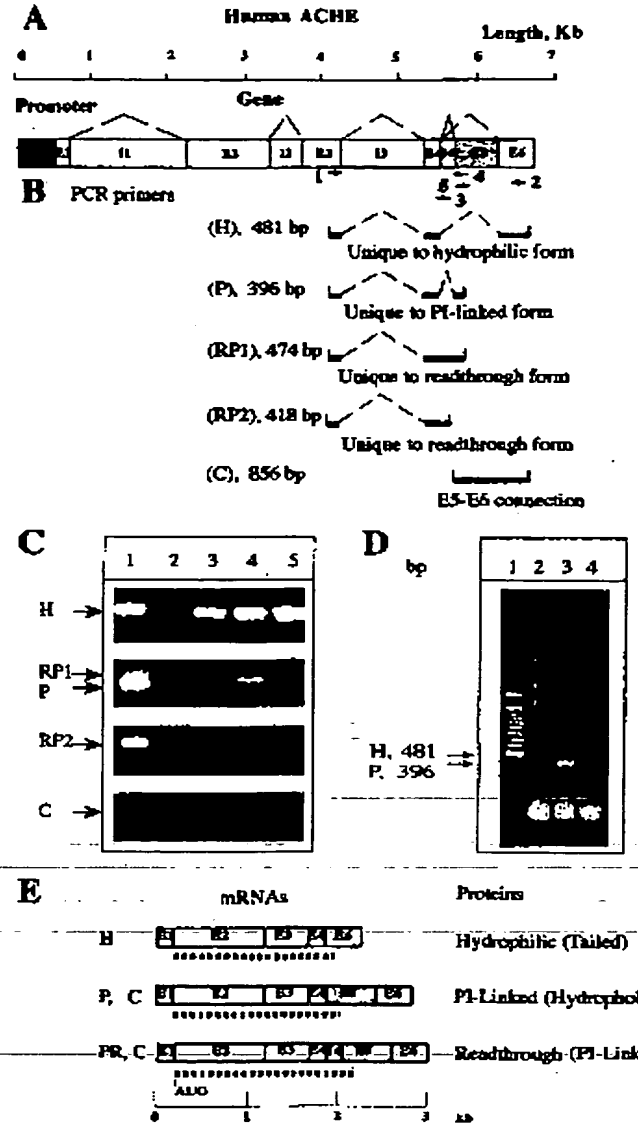

FIG. 3. Exon–intron organization and alternative options for tumorigenic expression in the human ACHE gene. (A) *Splicing patterns.* Splicing in the ACHE gene (scheme) is displayed by dashed triangles. Splicing of I1, I2, and I3 generates, in all tissues examined, the core domain of the coding sequence from exons E2–E4. Alternative splicing occurs in the I4, E5 region and includes three options: E4-E6, E4-E5,E6, and E4,I4,E5,E6. (B) PCR primer pairs and the selective RT-PCR products. PCR primers are numbered as described under Materials and Methods. The primer pair 1,2 could potentially create several alternative products, but practically it amplified only ACHE mRNA sequences including the E3, E4, and E6 regions, characteristic of the hydrophilic (H) form [15], with the potential for tailing [36]. This was probably due to unfavorable competition with the relatively more abundant major ACHE mRNA species. The primer pair 1,4 detected expression of the putative mRNA subtypes including the E5 exon which encodes PI-linked AChE (P) or the I4/E5 "readthrough" form of ACHE mRNA [16] encoding a longer PI-linked AChE (RP1). The primer pair 1,3 was unique to the readthrough (RP2) form, and the primer pair 2,5 amplified all ACHE cDNAs where the E5 exon in the ACHE gene is continued by E6 (C). (C) RT-PCR analysis of tumor cell lines. Cell lines were NT2/D1 teratocarcinoma [1], H9 T lymphoma [2], 293 embryonal kidney cells [3], NCI-N-592 small cell lung carcinoma [4], and TE671 medulloblastoma [5]. RT-PCR experiments were performed with 100-ng samples of total RNA and the noted primer pairs. Arrows indicate PCR products reflecting the various ACHE mRNA transcripts designated as in Fig. 3B. (D) Coexpression of common and alternative ACHE mRNAs in K-562 erythroleukemia cells. Total RNA from K-562 cells was subjected to RT-PCR amplification using the primer pairs 1,4 (lane 2,4) or 1,2 (lane 3). The lane 4 reaction was performed without reverse transcriptase, to exclude presence of genomic DNA contaminations. Molecular weight markers (Boehringer, Mannheim) were electrophoresed in parallel (lane 1). Arrows indicate PCR products and their sizes. (E) Predicted ACHE mRNAs and proteins. The three alternative ACHE mRNA transcripts and their putative protein products are schematically displayed. Open reading frames initiated by the AUG codon are marked by a dotted underline, all according to the bottom scale in kb. The resultant protein products would either be hydrophilic (H) and capable of being tailed by noncatalytic subunits or hydrophobic and amenable to linkage of phosphoinositide moieties (P or PR). In both latter cases, direct connection between E5 and E6 is predicted (C).

DISCUSSION

The findings presented in this report reflect a surprising complexity of alternative splicing patterns of ACHE mRNA transcripts in tumor cell lines from heterogeneous tissue origins. Furthermore, these variable ACHE mRNA species may encode three different AChE polypeptides, with potentially distinct properties, one of which is unique to humans.

Molecular Origin of Tumor ACHE mRNA Species

We found the dominant species of ACHE mRNA expressed in tumor cells to include exons E2, E3, E4, and E6. It encodes the globular hydrophilic AChE form [15], which may remain soluble [4, 36], interact with the collagen-like subunit characteristic of asymmetric AChE at the neuromuscular junction [43], or associate with a lipid-containing structural subunit in brain [44]. We further observed that the fourth intron, which follows the fourth exon, is variable in size within tumor cell lines. According to the dominant splicing pattern, this intron is 829 bp long, and its splicing connects the E4 and the E6 exons. Alternatively, the 3′-terminal 751 residues from this intron, or the entire 829 residues, are expressed and may directly be continued by the E6 exon. This leads to the production of the E5- or I4/E5-containing ACHE mRNAs. RT–PCR amplification was, not surprisingly, more sensitive than blot hybridization for detecting the alternative mRNA transcripts.

Variable AChE Polypeptides

Our findings predict the production of two forms of membrane-associated AChE from the alternative

TABLE 1

Expression of Alternative ACHE mRNA Transcripts in Different Tumor Cell Types

	(1) Source	Exons:	ACHE mRNA transcripts (2) Major E3, E4, E6	(3) E5 transcript E3, E4, E5	(4) Readthrough E3, E4, I4	(5) 3′ connection E5, E6	Other mRNAs (6) BCHE	(7) CHED
1.	Teratocarcinoma (NT2/D1)		10^6	++−	−+−	+	—	10^6
2.	T cell lymphoma (H9)		—	—	—	—	10^4–10^5	10^5
3.	Neuroblastoma (IMR32)		10^5–10^6	—	—		10^7	10^5
4.	Transformed embryonal kidney cells (293)		10^5–10^6	+	±	±		10^5
5.	Small cell lung Ca (NCI-N-592)		10^6	+	+	±	10^7	10^6
6.	Medulloblastoma (TE671)		10^6	+	+	±	10^5	10^6
7.	Chronic myelogenous leukemia K-562		+ +	+	nd	nd	nd	nd
8.	Promegakaryoblastic-DAMI		10^5–10^6	±	+	—	—	—
9.	Serous ovarian Ca.		++	—	—	—	nd	nd
10.	Mucinous cyst adenoma		++	—	—	—	nd	nd
11.	Papillary adenocarcinoma		±	—	—	—	nd	nd
12.	Uterine myoma		+++	—	—	—	nd	nd
13.	Fetal brain (21 W)		10^6	—	nd	nd	10^7	10^6
14.	Adult brain (70 Y)		10^5	—	nd	nd	10^6–10^7	10^4
15.	Adipose tissue		±	—	—	—	nd	nd

Note. (1) RNA was extracted from cultured cells, tumor biopsies, and normal tissues as detailed under Materials and Methods. Samples of 100 ng total RNA were subjected to RT–PCR amplification with the noted primer pairs. (2) Major ACHE mRNA transcripts containing E3, E4, and E6 (Fig. 1) were detected by the primer pair 1,2. (3) E5 containing ACHE mRNA was searched for using the primer pair 1,3, detecting transcripts including the E3, E4, and E5 regions. (4) Readthrough the ACHE mRNA was found with primer pair 1,4, detecting transcripts containing the E3, E4, and I4 regions. (5) The 3′-connection of alternative ACHE-mRNA-transcripts was examined by the primer pair 2,5, checking for the presence of the E6 exon-in-E5 containing-mRNAs. (6,7)-BCHE [31]-and CHED [32]-transcripts were amplified as detailed elsewhere (Ehrlich *et al.*, in preparation) to account for the integrity of the examined RNA preparations. Presented are copy numbers calculated for each transcript based on comparison with *in vitro* transcribed, deleted RNAs from each of the analyzed genes. Visual estimation of ACHE mRNA levels based on the intensity of PCR products at 39 cycles is presented, in decreasing order, as + + + (>10^6), ++ (10^5–10^6), + (10^4–10^5), ± (ca. 10^4), − (<10^4), nd, not determined.

ACHE-mRNAs in different human tumor cells, in addition to the hydrophilic form. Interestingly, the two hydrophobic peptides translated from these alternative ACHE mRNAs contain a free cysteine residue at the C-terminus, which implies that they both may be disulfide-linked to a second AChE monomer, to create the dimers characteristic of vertebrate erythrocytes [18]. That the alternative transcripts found in tumor cells are the molecular origin(s) for PI-linked AChE is indicated from reports that K-562 cells [18] are similar to various vertebrate erythrocytes [6] in their production of PI-linked AChE. It should be noted that in both the mouse and the rat ACHE genes, the I4 domain includes a termination codon [16, 42]. The inferred readthrough enzyme in human may hence be distinguished from the rodent enzymes both in its length (583 residues) and in its capacity for PI linkage. Yet, expression of ACHE mRNA does not necessarily imply production of its protein, as is indicated from the absence of AChE activity in 293 cells [45]. Elicitation of antibodies specific to the alternative peptides will therefore be required to reveal whether the inferred AChE forms are expressed in human tumor cells and to discover whether they may serve as tumor-specific markers. Peptide-specific antibodies will also reveal if the predominant E4/E6-containing ACHE mRNA species in K-562 cells is translated into an active hydrophilic protein.

Different choices of splicing options for ACHE mRNA may be physiologically important; thus, C-terminally mutated variants of the closely related human enzyme BChE display distinct differences in their inhibitor interactions compared with the normal enzyme [46]. This, in turn, suggests that an altered C-terminus may modify the biochemical properties of cholinesterases. AChE forms with apparently modified biochemical properties were, indeed, found associated with various tumor types [10; reviewed in Ref 2.] and in the demented brain of Alzheimer's disease patients [47]. One wonders whether alternative splicing could contribute to these modifications and to the distinct properties of embryonic AChE [3].

Putative Biological Implications

The question whether cholinesterases play a developmental role in tumorigenesis has been put forth by

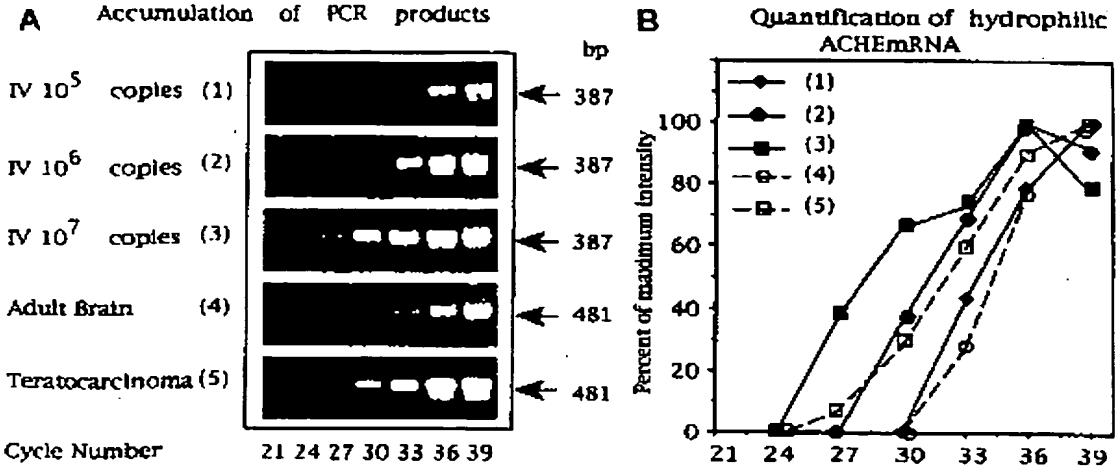

FIG. 4. Quantification of ACHE mRNA levels in teratocarcinoma cells and adult brain. (A) RT–PCR products. RNA samples (100 ng) extracted from adult human cortex or NT2/D1 teratocarcinoma cells were subjected to reverse transcription and PCR amplification as described under Materials and Methods. *In vitro* transcribed (IV) shorter PCR product was derived using the same primers from measured amounts of the deleted HdACHE mRNA (see Materials and Methods). Lengths of PCR products in base pairs (bp) are noted. (B) Photodensitometric measurement. Staining intensity for individual PCR products was quantified as detailed previously [33]. Maximal intensity within each experiment was taken as 100%. Relative intensity of the PCR products was plotted as a function of the number of PCR cycles at which samples were withdrawn.

many [reviewed in Ref 2.] and may be reevaluated in view of our present findings. Cholinesterase gene amplifications [3] have been correlated with a variety of tumors, including those of the nervous [34], reproductive [30], and hemopoietic systems [33]. However, the tumor-amplified ACHE gene tended to be incomplete [48] and therefore unlikely to drive effective transcription. Hence, it is not surprising that ACHE mRNA levels in the tumor cells were higher, yet within the same range as those in normal developing tissues. The presence of an E-box motif in the recently cloned ACHE promoter [35] suggests an alternative route for a more limited tumorigenic induction of ACHE, by the enhancement of transcription through c-*myc* [49]. Intensive transcription may thus explain the presently described alternative splicing patterns. This can occur by default, perhaps due to the tumorigenic lack of sufficient amounts of the specific protein factor(s) controlling the common splicing pattern of ACHE mRNA in brain. That transcription is particularly intensive in the tumor cell lines

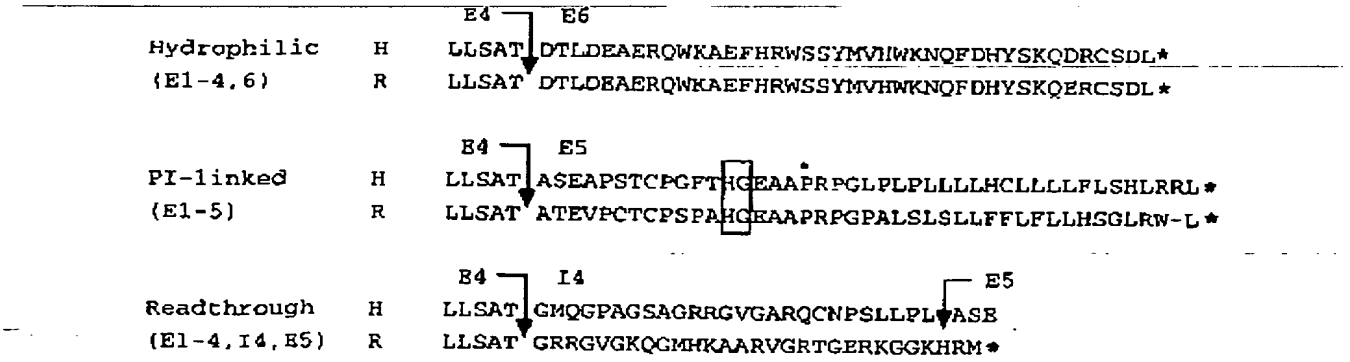

FIG. 5. Alignment of the variable translation products inferred from the alternative ACHEDNA sequences in human and rat. Amino acid sequences were deduced from the DNA sequence data as presented in the present and the previous report [15] for the alternative human AChE forms and as published [41, 42] for the counterpart rat AChE forms. Exon borders are delineated. The HG residues required for PI linkage are boxed. The proline residue which represents a natural polymorphism in the human sequence [38] is dotted. Note the conspicuous homologies between the E6 and E5 C-terminal peptides in human and rat and the absence of such homology for the I4-inferred readthrough peptide. Nonsense, termination codons are noted by asterisks.

is evident from the high levels of ACHE, BCHE, and CHED transcripts in them.

Interestingly, our findings demonstrate three alternative pathways for ACHE transcripts in tumor cell lines, yet not in primary tumor tissues. This may reflect mechanisms related with the mode of cell growth and which distinguish cultured cells from the *in vivo* situation. It should be noted in this respect that the ACHE promoter includes an Egr-1 motif, predicting serum induction [35]. Absence of angiogenic limitations under culture conditions can therefore upregulate ACHE mRNA transcription. Further studies should be performed to find out if this contributes to the alternative splicing and to reveal whether the predicted PI-linked AChE forms induce tumorigenic processes. The growth-regulatory role reported for AChE in murine erythroleukemic Friend cells [50] and observed recently by *in vivo* antisense inhibition of ACHE gene expression [51] is in line with this latter prediction. In conclusion, the pattern of AChE biosynthesis at multiple stages of the biosynthetic pathway presents an intricate model for the complex modulation of tumor-specific gene expression. Now that the necessary molecular tools and expression systems are available, integrated studies of these different levels of control in various tissues and stages of development may be instigated.

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